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The 63-Kilobase Circular Amplicon of Tunicamycin-Resistant *Leishmania amazonensis* Contains a Functional *N*-Acetylglucosamine-1-Phosphate Transferase Gene That Can Be Used as a Dominant Selectable Marker in Transfection

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Tunicamycin (TM)-resistant *Leishmania amazonensis* has been found previously to contain amplified chromosomal DNA, existing exclusively as extrachromosomal circles of 63 kb. Fragments of this DNA cloned into plasmids were functionally analyzed by transfection of wild-type cells. A clone with a 15-kb fragment of the 63-kb circle was initially found to confer TM resistance. A library of the 15-kb fragment was then prepared and used in toto to transfect wild-type cells. The transfectants that emerged after selection were found to contain a plasmid with an insert of 4.6 kb. Evidence from deletion experiments suggests that this is the minimal transfection-effective fragment. Sequencing of the 4.6-kb DNA revealed a 1.4-kb homolog of *N*-acetylglucosamine-1-phosphate transferase genes. The *L. amazonensis* gene is similar to those from two other sources in their deduced peptide sequence by 65 to 70% and in hydropathic characteristics. The *L. amazonensis* gene is amplified by more than 128-fold over the wild type and overproduces a major transcript of 2.4 kb in all transfectants. The endogenous copy of this gene was amplified by polymerase chain reaction from the wild type and cloned into pX-NEO, a *Leishmania* expression vector. Amplification of this plasmid in the transfectants by selection with G418 simultaneously made them resistant to TM. Evidence provided thus indicates that the 1.4-kb DNA is an *N*-acetylglucosamine-1-phosphate transferase gene whose amplification is responsible for TM resistance in *Leishmania* variants and transfectants.

Gene amplification is a well-known mechanism of drug resistance in many types of cells and organisms (36). Much of the work has been done with mammalian cells, in which the gene of relevance is often amplified along with a very large chromosomal region (13). The amplification of this gene results in overproduction of its product, allowing cells to overcome the drug inhibition (31).

Recently, DNA amplification has been found to occur frequently in *Leishmania* spp. during in vitro selection of these trypanosomatid protozoa for drug resistance (4). The amplicons are derived from different chromosomal regions, depending on the drugs used for the selection. The amplified chromosomal DNA often exists exclusively as multiple copies of extrachromosomal circles. A single gene within the circle is usually responsible for drug resistance. *Leishmania* genes amplified in this way in drug-resistant cells include, for example, those encoding dihydrofolate reductase-thymidylate synthase (9), *p*-glycoproteins (28), and inosine monophosphate dehydrogenase (39). The frequent occurrence of DNA amplification, coupled with the small genome size of *Leishmania* spp., makes this group of organisms favorable for studying this phenomenon.

Previously, we described DNA amplification in *Leishmania amazonensis* (*Leishmania mexicana amazonensis*) made resistant to tunicamycin (TM) (18), an antibiotic, which preferentially inhibits *N*-acetylglucosamine-1-phosphate transferase (NAGT) in the dolichol pathway of glycosylation (38). Initially, three lines of TM-resistant variants were independently produced by gradual acclimatization of wild-type cells to increasing drug concentrations. The amplified DNA in all cases was found to originate from a large chromosome, existing extrachromosomally as 63-kb supercoiled circles (12). With increasing drug concentrations, this circular DNA increases in copy number in the variants, as do their NAGT activities and protein glycosylation (19, 20). Twelve additional variants were subsequently produced from four other *Leishmania* species, all of which were found to contain circular amplicons. These amplicons vary with different species from 30 to 70 kb in size, but all share a consensus region of 20 kb (17). We have suggested from these studies that gene amplification is the predominant, if not the only, mechanism of TM resistance in *Leishmania* spp. However, attempts to map the relevant gene in the amplicon by using Alg7, the *Saccharomyces* NAGT gene (14), were unsuccessful because of poor homology between yeast and *Leishmania* DNAs.

We report here the use of an alternative strategy to map the functional gene of the amplicon from TM-resistant *Leishmania* spp. Fragments of the extrachromosomal amplified DNA were used for transfection of wild-type cells. Leishmanial DNA relevant to TM resistance was recovered from the plasmids found in the transfectants selected with the antibiotic. By this approach, coupled with the use of deletion clones, a region of 4.6 kb within the 63-kb circle was defined as the minimal effective fragment. In this region, we further identified a 1.4-kb open reading frame (ORF), which resembles NAGT genes from yeast and CHO cells. All effective plasmids with the *Leishmania* gene, irrespective of their insert size, are amplified to the same level in the transfec-

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tants. All these transfectants also overproduce a major NAGT transcript of identical size. The wild-type copy of this gene is functionally effective when amplified by polymerase chain reaction (PCR) and cloned in a *Leishmania*-specific expression vector. The results obtained clearly indicate that the amplification of the NAGT gene mediates TM resistance of *Leishmania* spp., as previously proposed (19).

MATERIALS AND METHODS

Cells. Wild-type and TM-resistant *L. amazonensis* (LV78) promastigotes were grown as before (19).

Transfection of *Leishmania* spp. by electroporation. Wild-type cells were transfected with plasmids by electroporation as previously described (16, 24). Briefly, promastigotes at late log or stationary phase were washed twice and resuspended to 10^8/ml in ice-cold electroporation buffer (21 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 137 mM NaCl, 5 mM KCl, 0.7 mM NaH_2PO_4, 6 mM glucose). The cell suspension (0.3 ml) was transferred to a 0.4-ml electroporation cuvette (Bio-Rad) with 5 to 20 μg of chilled DNAs. Cells were electroporated in Gene Pulser (Bio-Rad) at 0.45 kV and 500 μF. After recovery in 3 ml of fresh medium for 6 to 9 h, each lot of the electroporated cells was selected with three concentrations of TM, i.e., 5, 10, and 20 μg/ml. It is known from previous work that the wild-type cells are sensitive to these concentrations of TM. Cells were monitored for viability and growth by microscopy. The level of TM resistance was assessed by determining the 50% effective doses (ED_{50}s) of TM for growth inhibition as before (18).

DNA isolation, electrophoresis and hybridization conditions. Genomic and plasmid DNAs were isolated from *L. amazonensis* as described before (17). Previously, the extrachromosomal circular DNA of 63 kb found in TM-resistant variants has been cloned in pBR322 as four *Bam*HI fragments of 12, 14, 15, and 22 kb (pTR12, pTR14, pTR15, and pTR22, respectively) (12). These and other plasmids were isolated from bacteria by CsCl-ethidium bromide gradient ultracentrifugation (2). For Southern analysis, electrophoresed DNAs were transferred to Zetabind (CUNO), as recommended by the manufacturer. Probes were ^{32}P labeled by using random hexanucleotide primers. Hybridization was performed in the presence of heparin as previously described (35). Filters were washed twice briefly at room temperature and twice at 65°C each for 15 min with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1× sodium dodecyl sulfate (SDS). Samples for orthogonal pulsed-field agarose gel electrophoresis (OFAGE) were prepared as before (12). Electrophoresis was run at a constant voltage of 300 V with a pulse interval of 40 s for 20 h.

Northern (RNA) blot analysis. Total RNAs were isolated from *L. amazonensis* as described before (17). Denatured RNAs were electrophoresed in 1% agarose gel with formaldehyde and blotted onto nitrocellulose paper. Hybridization was performed at 42°C in the presence of 5× Denhardt's solution–50% formamide–5× SSC–0.5% SDS–150 μg of herring sperm DNA per ml (2). Blots were washed at 42°C for 1 h with 2× SSC–0.5% SDS.

Subcloning of the amplified DNA for library construction and DNA sequencing. Standard procedures were used for DNA cloning and sequencing (2). For library construction, the 15-kb *Bam*HI fragment of pTR15 was partially digested with 1 U of *Pst*I per μg of DNA for up to 10 min. One μg of this DNA sample was ligated to 500 ng of *Pst*I-digested and dephosphorylated pUC19. The ligation mixture was used to transform *Escherichia coli* (DH5α). About 300 white colonies were randomly picked and pooled to form the library. For sequencing, DNA fragments were cloned into replicative forms of M13mp18 and M13mp19. Nested deletion clones were made by Cyclone I Biosystem (IBI). Sequencing was performed by the dideoxynucleotide chain termination method (30) with Sequenase (USB). The majority of the sequences was obtained with the −40 primer, except for two regions of about 200 bp each, which were sequenced by using designed oligo primers. The DNA was sequenced completely in both directions. DNA sequence was analyzed by the University of Wisconsin Genetics Computer Group software package, version 7.0.

PCR amplification. Oligo primers were synthesized for PCR amplification of the NAGT gene from wild-type *Leishmania* DNAs. Standard procedures recommended by the manufacturer (GeneAmp; Perkin-Elmer) for PCR were followed. Sixty nanograms of template DNA was used. Reactions were carried out in a DNA Thermal Cycler (Perkin-Elmer Cetus) for 1 min each at 94, 55, and 72°C per cycle for a total of 35 cycles.

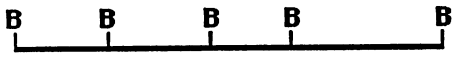
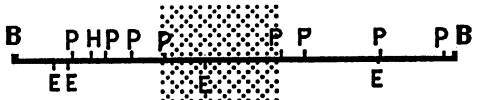
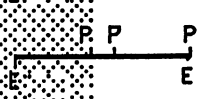
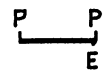
Nucleotide sequence accession number. The GenBank nucleotide accession number for the 4.6-kb DNA is M96635.

RESULTS

Electroporation of wild-type cells with the 63-kb amplicon and its large fragments for selecting TM-resistant transfectants. We began this work with the 63-kb DNA circles from TM-resistant variants of *L. amazonensis* and its four large *Bam*HI fragments of 12, 14, 15, and 22 kb cloned in pBR322, i.e., pTR12, pTR14, pTR15, and pTR22, respectively (12). These four plasmids and the complete 63-kb circles were functionally analyzed for their ability to confer TM resistance by transfection of wild-type cells. Cells were transfected by electroporation and then selected with TM under conditions described in Materials and Methods. The transfectants electroporated with the complete 63-kb circles or pTR15 survived the selection, while those with the other three plasmids containing the remaining portion of the circles did not (Table 1). All transfectants decreased in viability initially, but a small number of survivors among those transfected with pTR15 or the 63-kb circles began to grow in 1 to 2 weeks. These transfectants grew during the subsequent passages in TM-containing medium as well as the parental wild-type cells in drug-free medium. In more than 20 separate experiments, transfectants with pTR15 have been successfully selected in this way with initial TM concentrations of 5 to 10 μg/ml. These transfectants became adapted to grow readily without delay in media with higher TM concentrations of up to 80 μg/ml. In contrast, no survivors emerged among cells electroporated with pTR12, pTR14, pTR22, or pBR322 under the same selective conditions. Viable cells were not seen in these cultures despite several renewals of medium during a period of up to 30 days. Transfection of other *Leishmania* spp., e.g. *L. tropica*, with pTR15 was also successful (data not shown).

The ED_{50} of TM for growth inhibition was 100 μg/ml for the transfectants made resistant to 10 μg of TM/ml, comparable to that of the variants stepwise selected previously from wild-type cells for resistance to the same TM concentration (19); both ED_{50}s were about 25-fold higher than that of the parental wild-type cells (Fig. 1). The comparable degree of TM resistance between the transfectants and the variants indicates that the 15-kb insert in pTR15 is functionally as efficient as the complete 63-kb circles. The results

TABLE 1. Functional mapping of the 63-kb amplicon for TM resistance gene by transfection of wild-type cells

Plasmid[a]	Insert map[b]	Insert Size (kb)	No. Exp.	Tunicamycin resistance[c] (5–20 μg/ml)
ECC63		63	2	+
pTR14		14	3	–
pTR12		12	3	–
pTR22		22	3	–
pTR15		15	20	+
pTR15		15	20	+
pTR15H		12	2	+
pUCP5.2		5.2	3	+
pUCP4.6		4.6	3	+
pUCE5.0		5.0	2	–
pUCE6.6		6.6	2	–
pUCP2.7		2.7	3	–

[a] ECC63, the complete 63-kb extrachromosomal circles isolated from TM-resistant variants of *L. amazonensis*; pTR12, -14, -15, and -22, *Bam*HI fragments of the 63-kb amplicon cloned in pBR322; pTR15H, pTR15 with deletion by digestion with *Hin*dIII; pUCE and pUCP, *Eco*RI and *Pst*I fragments of pTR15 cloned in pUC19.

[b] B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII. P, *Pst*I; shaded area, functionally active region of 4.6 kb with TM resistance gene.

[c] Wild-type cells were transfected with plasmids by electroporation and selected separately with 5, 10, and 20 μg of TM per ml. +, transfectants that survived the selection and grew during subsequent passages in TM-containing medium.

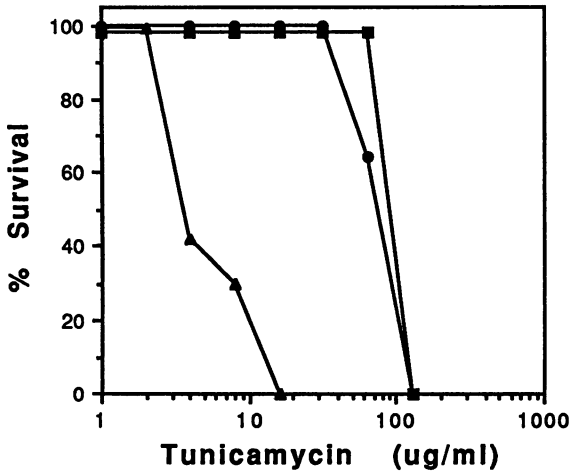


FIG. 1. TM sensitivity of wild-type cells, TM-resistant variants, and transfectants. Cells grown to late log phase were resuspended to a cell density of 5×10^6/ml in culture medium with different concentrations of TM as indicated. The cell number in each culture was counted on day 5 when stationary phase of growth was reached. The percent survival was determined from the ratio of the cell number in a given drug concentration to that in drug-free medium. ▲, parental wild type; ●, variant resistant to 10 μg of TM per ml; ■, transfectants with pTR15 selected with 10 μg of TM per ml.

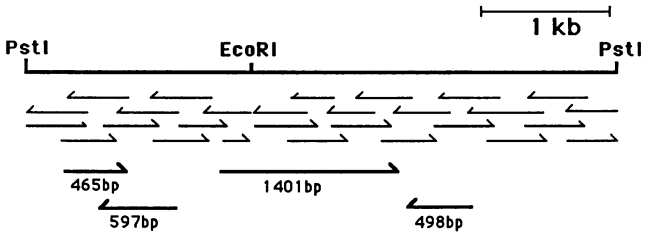


FIG. 2. Map of the deletion clones for sequencing the 4.6-kb *Leishmania* DNA. Fragments of the 4.6-kb DNA were subcloned into M13mp18 or M13mp19. Nested deletion clones were prepared by using Cyclone I Biosystem (IBI). Sequencing was done by the dideoxyribonucleotide termination method with Sequenase (USB). Thin lines with arrows, sequences obtained from individual clones; thick lines with arrows, open reading frames larger than 450 bp.

thus suggest that the genetic elements crucial for TM resistance are contained within the 15-kb region of the 63-kb circles.

Delineation of the minimal DNA sequence for TM resistance by functional analysis. The 15-kb insert of pTR15 was further analyzed to define the smallest functional region of this DNA for TM resistance. On the basis of the restriction map constructed previously (12), several larger fragments of the 15-kb insert were subcloned into pUC19. Functional analysis of these clones by transfection of the wild-type cells initially identified the central portion of the 15-kb insert with an *Eco*RI site as a region of importance. For example, deletion of a 3-kb *Bam*HI-*Hin*dIII fragment from one end of the 15-kb insert (pTR15H) had no adverse effect, whereas separate clones with the two large *Eco*RI fragments (pUCE5.0 and pUCE6.6) lost activity completely (Table 1).

Further studies were done with the library constructed in pUC19 with *Pst*I partial digests of the 15-kb DNA. All the *Pst*I fragments of expected sizes were found in the library. The wild-type cells were first electroporated with this DNA library in toto and then selected for TM resistance under the conditions described above. The transfectants emerged in 20 days and were found to contain a single pUC19 clone with a 4.6-kb insert. The insert was mapped precisely to the 4.6-kb *Pst*I fragment of pTR15 by Southern hybridization (cf. Table 1). The library contained several functional clones with larger inserts inclusive of the 4.6-kb DNA. The replicative advantage of smaller plasmids over the larger ones may explain the emergence of only the clone with the 4.6-kb insert in the transfectants. We further isolated this and other clones from the library, i.e., pUCP2.7, pUCP4.6, and pUCP5.2 (which contained *Pst*I fragments of 2.7, 4.6, and 5.2 kb of the 15-kb DNA, respectively) (see map in Table 1). Transfection of wild-type cells with these plasmids individually revealed that TM resistance was conferred to the wild-type cells by pUCP4.6 and pUCP5.2 but not by pUCP2.7 (Table 1). pUCP4.6 was found to lose activity completely when the 4.6-kb insert was further shortened. It is clear from additional studies that the 4.6-kb DNA is very close to the minimal size with full activity, although its exact size remains to be mapped.

Comparative studies of pUCP4.6 and pTR15 showed that they were equally effective, as assessed by several criteria, e.g., the period of selection needed for the emergence of the transfectants, the level of TM resistance achieved, and the plasmid copy number in the transfectants (see Fig. 10, cf. lanes 2 and 3). All the essential elements for TM resistance are thus further localized to a 4.6-kb region of the 15-kb DNA.

Identification of NAGT gene in the 4.6-kb minimal effective region by DNA sequencing. The 4.6-kb DNA was completely sequenced (data not shown) to identify the gene relevant to TM resistance. This DNA was first subcloned into M13 mp18/M13mp19 as two *Eco*RI-*Pst*I fragments of 2.85 and 1.75 kb (Fig. 2). The inserts were progressively deleted with T4 DNA polymerase to generate a sufficient number of clones, from which overlapping sequences of both strands were obtained to cover the entire length of the 4.6-kb DNA (Fig. 2). Sequence analysis of this DNA revealed a large ORF of 1.4 kb and three smaller ones of less than 600 bp each (Fig. 2). The GenEMBL data base was searched for homologous genes of these ORFs. The 1.4-kb gene (Fig. 3) was found to have similarity with two sequences, i.e., TRG-22/3E (33, 40) and Alg7 (14), which are predicted to code for the NAGT of CHO cells and yeast cells, respectively (see below for detailed sequence comparison). No homologous genes were found for the remaining three small ORFs.

Sequence comparison of the NAGT genes from *L. amazonensis* and other eukaryotic cells. Figure 3 shows the 1.4-kb ORF and its deduced amino acid sequence within the 1259- to 3025-nucleotide region of the 4.6-kb DNA. The nucleotides are numbered with reference to the first base at the *Pst*I site on the left end of the 4.6-kb fragment (Fig. 2). The 1.4-kb ORF shows similarity with Alg7 (14) and TRG-22/3E (33, 40), the only two NAGT genes sequenced thus far. The *L. amazonensis* gene has two in-frame ATG codons at its 5′ end. We assign the first one as the initiation codon based primarily on the best alignment with the other two gene sequences. The size of the polypeptide deduced from the *L. amazonensis* gene is 51.4 kDa, consistent with that of NAGT purified from the bovine mammary gland (34). The next closest ATG in frame is located 297 bp downstream (Fig. 3). The selection of this initiation codon would make the size of

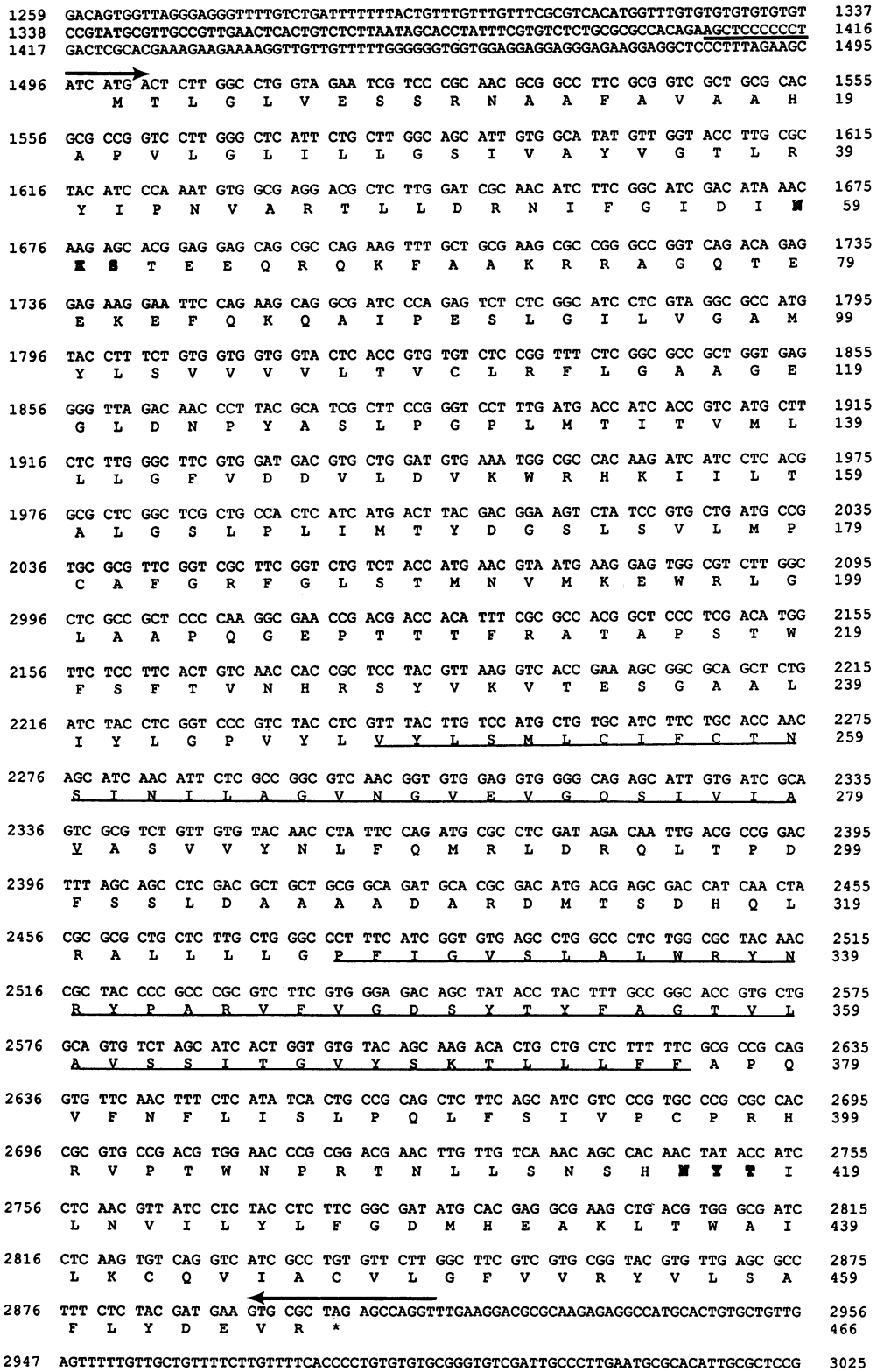


FIG. 3. Nucleotide and amino acid sequence of the 1.4-kb ORF. Boldface residues, potential sites of *N*-glycosylation; *, termination codon; underlines, two regions of highest similarity with the NAGT genes from yeast and CHO cells; overlines with arrows, sequences of the oligos used as primers for PCR amplification of this gene.

Region I

```
CHO  171 VYMGLLAVFCINAINILAGINGLEAGQSLVISA 203
         ||:::|.:|||||.|||||:|||:|.|||:||..
La   248 VYLSMLCIFCINSINILAGVNGVEVGQSIVIAV 280
         ||:. :.|||.|||||||||||:||||:||:|:
Alg7 203 VYMASMAIFCPNSINILAGVNGLEVGQCIVLAI 235
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Region II

```
CHO  230 PFFFTTLGLLYHNWYPSQVFVGDTFCYFAGMTFAVVGILGHFSKIMLLFF 279
         ||: ..|:|: .|:||..|||||.:.|||| .:|| :| | :|||:||||
La   327 PFIGVSLALWRYNRYPARVFVGDSYTYFAGIVLAVSSITGVYSKTLLLFF 376
         ||:|||||||::||:|| |||||.|.|||| |:|| :| | :|||:||:|
Alg7 265 PFLGVSLALWKWNRWPATVFVGDTYCYFAGMVFAVVGILGHFSKIMLLLF 314
```

FIG. 4. Peptide sequence alignment of the two most similar regions within NAGT genes of yeast cells, CHO cells, and *Leishmania* spp. The alignment was generated by the University of Wisconsin Genetics Computer Group Gap program. The amino acid residues are numbered with reference to their respective translational initiation codons. Alg7, yeast sequence; CHO, CHO cell sequence; La, *Leishmania* sequence; single dot, amino acid residues of limited similarity; colon, amino acid residues of substantial similarity; vertical line, identical amino acid residues.

the *L. amazonensis* enzyme smaller than those predicted from the genes of other sources.

Sequence alignment of the *L. amazonensis* gene with Alg7 and TRG-22/3E indicates that they share 65 to 70% similarity at the amino acid level. Identity and conserved substitution account for ~32% and ~21% of the amino acid residues, respectively. The similarity is only 45% at the nucleotide level. This discrepancy is not unexpected in view of the known G/C codon bias of *Leishmania* DNA at the third position of the triplet codon (5, 6). This bias reaches a level of more than 75% in the *L. amazonensis* gene (Fig. 3). The three genes share the highest similarity of 80 to 90% in two regions, consisting of 33 and 50 amino acid residues, respectively (Fig. 4). The sequence conservation in these two regions among the three evolutionarily divergent species implies their functional or structural importance. Interestingly, these two regions do not include the dolichol recognition site (1)—a sequence of 13 amino acid residues (LFVXFXXIPFXFY) predicted by comparing the genes of three yeast glycosyltransferases known to interact with dolichol phosphate. The NAGT gene from CHO cells contains two such sequences, but the assignment in this case requires a reduction of the consensus to 11 residues (FI/VXF/YXXIPFXF/Y) with a high degree of degeneracy (40). None of these sequences is present in the *L. amazonensis* gene. However, two hydrophobic regions of amino acid residues 101 to 111 and 319 to 331 in this gene (Fig. 3), i.e., LSVVVVLTVCL and LRALLLLGPFIGV, correspond in position to the consensus when optimally aligned by BEST FIT with the CHO gene. Despite their poor similarity to the consensus, these sequences are extremely hydrophobic, consistent with the dolichol-binding property.

Hydropathy analysis (23) of the peptide sequence deduced from the *L. amazonensis* gene indicates that the NAGT has a high index of hydrophobicity (Fig. 5), suggestive of its membrane-bound nature. The hydropathy plots of the peptides from the three NAGT genes are very similar (Fig. 5), predicting the presence of about 10 transmembrane domains. The two best-conserved regions (Fig. 4) have the most similar hydropathic characteristics (Fig. 5, shaded boxes). The *L. amazonensis* gene has two potential *N*-glycosylation sites (Fig. 3), which appear to reside on two hydrophilic domains. In contrast, the sequence from the CHO gene predicts four potential *N*-glycosylation sites present on both sides of the membrane (40).

Overexpression of NAGT transcripts in the transfectants. Northern blot analysis was performed to study the NAGT gene transcripts. The coding region of the gene was amplified by PCR and used as a probe (see PCR details below). Hybridization revealed a major band of the same size (2.4 kb) in RNA samples from transfectants with pUCP4.6, transfectants with pTR15, and the TM-resistant variants (Fig. 6A). This band is visible in wild-type samples, but only after prolonged exposure of the blot (data not shown). Two minor bands of ~1.8 and ~4.0 kb were also present (Fig.

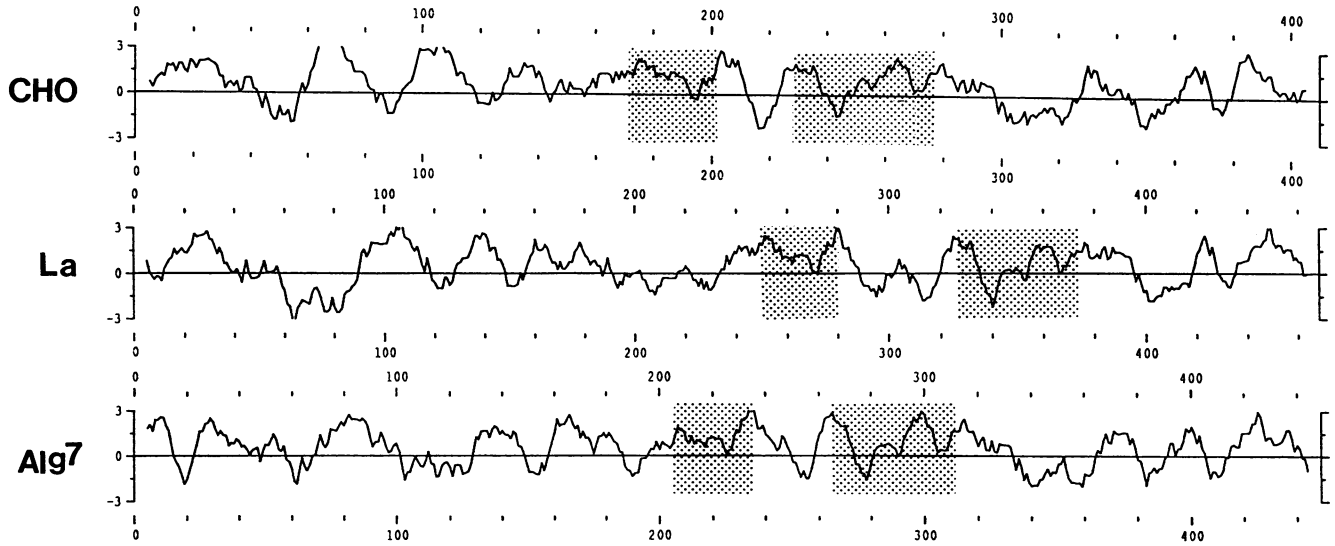


FIG. 5. Hydropathy plots of the deduced NAGT gene products from three different organisms. The plot was generated by "Pepplot" of the University of Wisconsin Genetics Computer Group program based on the method of Kyte and Doolittle (23). The shaded boxes represent the two regions of highest homology presented in Fig. 4.

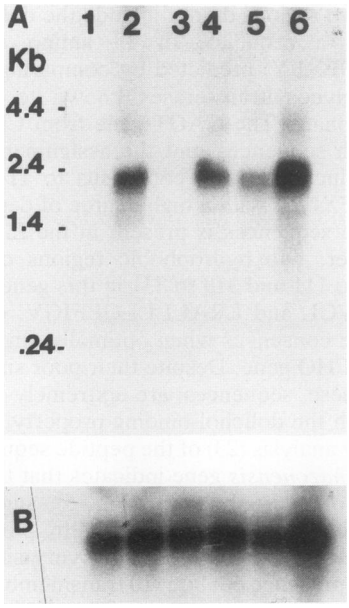


FIG. 6. Northern blot analysis of the NAGT transcripts. Total cellular RNA from wild-type cells (lanes 1 and 3), transfectants with pUCP4.6 (lane 4), transfectants with pTR15 (lane 2 and 5), and TM-resistant variants (lane 6) was electrophoresed and transferred to a nitrocellulose filter. The filter was probed with the PCR-amplified 1.4-kb NAGT gene (panel A) and beta-tubulin gene (panel B).

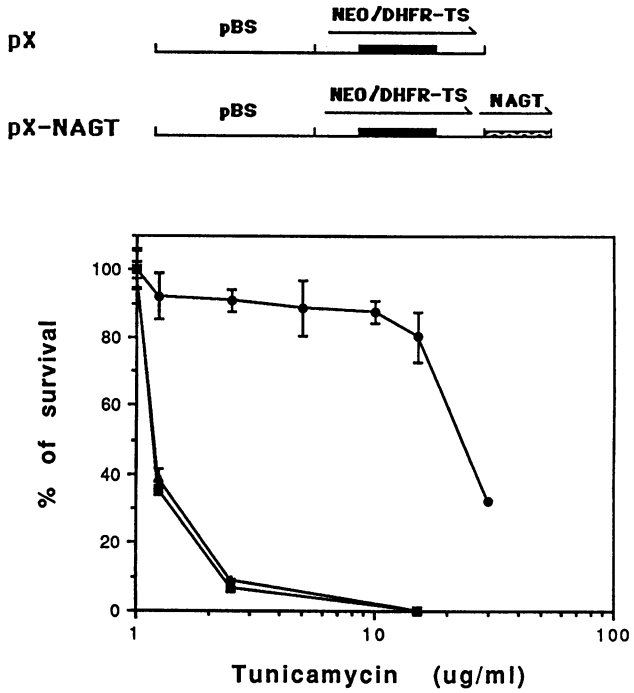


FIG. 7. Linear map of pX-NAGT and TM resistance of the transfectants with this plasmid. (Upper panel) Schematic drawing of the pX and pX-NAGT construct. The 1.4-kb NAGT gene amplified from wild-type total DNAs by PCR was inserted downstream of the NEO/DHFR-TS fusion gene. (Lower panel) Cells transfected with pX and pX-NAGT were selected for G418 resistance. The resulting transfectants and parental wild-type cells were examined for TM resistance as described in the legend to Fig. 1. ■, wild-type cells; ▲, cells transfected with pX; ●, cells transfected with pX-NAGT.

6A). The *Leishmania* NAGT transcripts are larger than those of the corresponding species previously described, i.e. 1.38 and 1.56 kb in yeast cells (21), and 1.5 to 2.2 kb in CHO cells (26, 33). When normalized against signals for tubulin gene on the same blot (Fig. 6B), the transcriptional levels of the *Leishmania* NAGT gene appeared very similar among all the TM-resistant cells. This gene thus appears to function equivalently in different transfectants with plasmids or extrachromosomal circles of very different sizes. More significantly, the results indicate that the NAGT gene is not only amplified but also overexpressed in the transfectants.

TM resistance of G418-selected transfectants with the NAGT gene in pX vector. The functional significance of the NAGT gene was further verified by cloning it in pX, a *Leishmania* expression vector (25). This vector uses Neo^r as the selective marker and the flanking regions of dihydrofolate reductase-thymidylate synthase gene as the regulatory elements. Foreign genes have been successfully expressed in pX. The NAGT gene was cloned into this vector so that its activity to confer TM resistance could be assessed without prior exposure to this drug and in the absence of the flanking sequences in the 4.6-kb DNA. The 1.4-kb coding region of the NAGT gene was amplified from the wild-type DNA by PCR. The two primers were synthesized according to the sequences at the N- and C-terminal ends of the gene, i.e., GGAG*GGATCC*TTTAGAAGCATCATGA and TGC*GGATCC*TTCAAACCTGGCTCTAGCGCAC (Fig. 3, overlines with arrows). Both primers were modified at the 5′ end to create a *Bam*HI site to facilitate cloning. As expected, the amplified products appeared as a single band of 1.4 kb and hybridized with the 4.6-kb fragment from pUCP4.6 (data not shown). The 1.4-kb amplified product was cloned into the pX vector at the *Bam*HI site behind the Neo^r in the same orientation to produce a new plasmid, pX-NAGT (Fig. 7, upper panel). Transfectants with this plasmid and those with pX were selected for resistance to G418. Both transfectants survived and grew equally well in medium with 10 μg of G418 per ml, a drug concentration cytotoxic to the parental wild-type cells. Both transfectants were then tested for TM resistance. Determination of their ED_{50}s showed that the transfectants with pX-NAGT were ~24-fold more TM-resistant than the transfectants with pX alone or the parental wild-type cells (Fig. 7, lower panel). There was clearly no contribution of endogenous gene amplification to the phenotype, as these transfectants were not exposed to TM before the assessment of their TM resistance. This demonstration of the NAGT gene activity in pX indicates that this gene is the functional element directly responsible for TM resistance in the 4.6-kb DNA (Table 1B), independent of other small ORFs also present in this minimal effective fragment (Fig. 2, thicker arrows). The origin of the gene in pX-NAGT from wild-type DNA also provides evidence indicating that the endogenous copy is functionally identical to those amplified in the TM-resistant transfectants and variants.

Plasmid DNAs in TM-resistant transfectants. The plasmids remained unchanged in the transfectants as extrachromosomal circles. In an ethidium bromide-stained OFAGE gel, both TM-resistant transfectants (Fig. 8A, lanes 2, 6, and 7) and variants (lanes 1 and 5) were found to contain extrachromosomal DNA circles (arrows) absent in the wild type (lane 3). These circular DNAs migrated in a characteristic manner different from that of the linear chromosomal bands (3, 8). Hybridization of pBR322 with the circular DNA bands

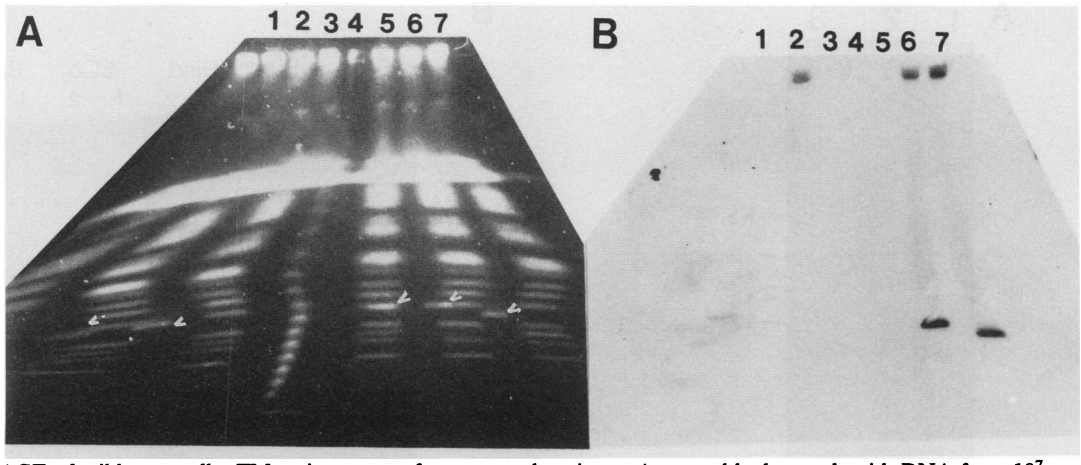


FIG. 8. OFAGE of wild-type cells, TM-resistant transfectants, and variants. Agarose blocks, each with DNA from 10^7 promastigotes, were electrophoresed at 300 V with a 40-s pulse interval for 20 h. Lanes: 1, TM-resistant variants; 2, transfectants with pTR15 selected for resistance to 10 μg of TM per ml; 3, parental wild-type cells; 4, lambda ladders as molecular weight markers; 5, cells transfected with the 63-kb circles; 6 and 7, cells transfected with pTR15 and pTR15H (Table 1), respectively, and selected for resistance to 80 μg of TM per ml. (A) Ethidium bromide-stained gel; (B) blot of the gel probed with labeled pBR322. Extrachromosomal supercoiled circular DNAs (arrows) migrate in a path straighter than the linear chromosomal DNAs. As expected, pBR322 hybridizes only with pTR15 and pTR15H from the transfectants (lanes 2, 6, and 7).

(Fig. 8B, lanes 2, 6, and 7) indicated that pTR15 retained this configuration in the transfectants. The circular DNAs in the variants (lanes 1 and 5) contained no pBR322 sequence, accounting for the lack of hybridization. Transfectant DNAs also gave strong hybridization signals in the wells (lanes 2, 6, and 7) where relaxed or concatenated circles were presumably trapped (3, 15). The hybridization signals were, as expected, stronger with samples from transfectants resistant to 80 μg of TM per ml (Fig. 8B, lanes 6 and 7) than with samples from those resistant to 10 μg of TM per ml (lane 2).

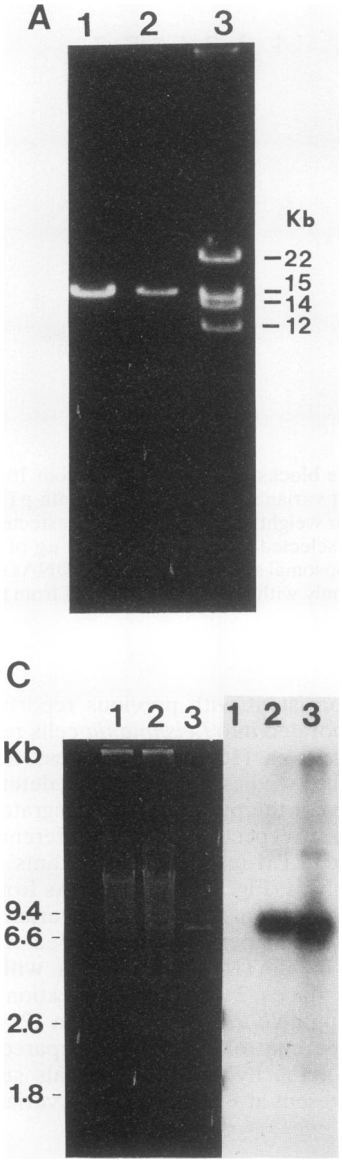
Extrachromosomal circular DNAs were isolated by alkali lysis from transfectants and *E. coli* with pTR15 and from TM-resistant variants with the 63-kb circles for further studies. Digestion of these three DNA samples with *Bam*HI yielded fragments of expected numbers and sizes, i.e., fragments of 12, 14, 15, and 22 kb from the 63-kb circles of the variants (Fig. 9A, lane 3) and only the 15-kb *Leishmania* DNA plus the 4.4-kb pBR322 from the transfectants and *E. coli* (lanes 1 and 2). Mapping of the latter two plasmids with three different restriction enzymes further demonstrated their identity (Fig. 9B). Thus, the pTR15 plasmid is structurally unaltered in the transfectants. Also, the samples from the *Leishmania* transfectants contained no additional *Bam*HI fragments of the 63-kb circles (Fig. 9B), indicating the absence of endogenous chromosomal DNA amplification in these cells.

There is no apparent evidence for integration of electroporated plasmids into the chromosomes, although this possibility cannot be totally eliminated. In the OFAGE blot (Fig. 8), we observed no hybridization of pBR322 with any OFAGE-resolvable linear chromosomal bands of the cells transfected with pTR15 even after prolonged exposure (data not shown). Mapping the plasmid DNAs in the total DNAs of the transfectants supports this point. *Bam*HI cuts pUCP4.6 only once in the vector and thus linearizes this plasmid into a single band of 7.3 kb. Only this *Bam*HI fragment and a trace of uncut plasmid were seen in both plasmid and transfectant DNA samples (Fig. 9C, lanes 2 and 3). Additional bands in the latter sample would be expected in the event of extensive chromosomal integration. Our findings are consistent with previous reports that plasmid DNAs electroporated into *Leishmania* cells remain as extrachromosomal circles (16, 24, 25) unless linearized (10). However, further studies are needed to determine whether very few copies of the plasmid have integrated.

All plasmids, irrespective of their difference in size, are highly amplified in TM-resistant transfectants, as determined by dot blot analysis (Fig. 10). Total DNAs from the parental wild type, different transfectants, and variants made resistant to 80 μg of TM per ml were probed with the 1.4-kb NAGT gene. In all TM-resistant cells with plasmids of different sizes (lanes 2 to 4), amplification of this gene reached a comparable level of more than 128-fold over that of the wild-type control (lane 1). Compared with that of pTR15 (lane 5), the hybridization signals suggest that all plasmids are present at ~300 copies per variant or transfectant cell.

DISCUSSION

We have demonstrated in the present study that DNA amplification is directly related to TM resistance in *Leishmania* spp. by functional identification of the responsible gene within the amplified sequence. This is supported by our previous findings that DNA amplification is associated with an elevated microsomal NAGT activity of the TM-resistant variants (18) and the presence of a consensus amplified region (17). We now provide direct proof that a 4.6-kb region of the 63-kb amplicon confers TM resistance when used to transfect wild-type cells (Table 1). Complete sequencing of the 4.6-kb DNA further reveals a 1.4-kb gene homologous to those encoding NAGT (Fig. 2 to 5). Several lines of evidence are presented, indicating that amplification of this gene alone in the transfectants is responsible for their TM resistance. The NAGT gene is highly amplified (Fig. 10) and overexpressed (Fig. 6) to a level comparable among different transfectants, regardless of whether they contain the complete 63-kb circle or its deletion clones of variable sizes, as long as the 4.6-kb region is included. Thus, the DNA sequences other than this region in the 63-kb circle appear to



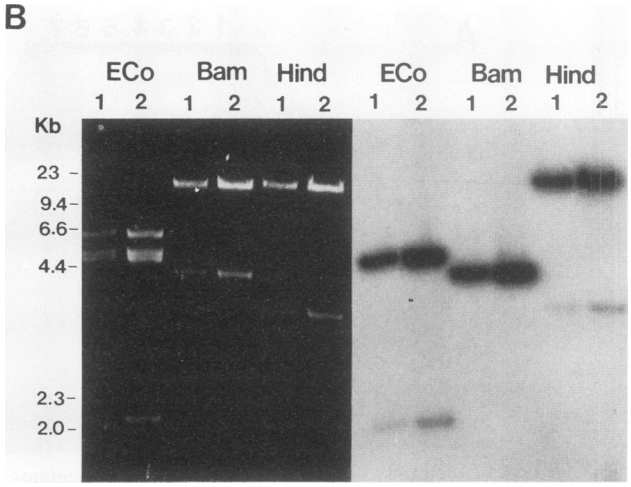


FIG. 9. Plasmids from *Leishmania* transfectants. (A) *Bam*HI fragments of pTR15 from transfectants. Lane 1, pTR15 plasmids isolated from the transfectants; lane 2, pTR15 purified from *E. coli*; lane 3, the 63-kb circular amplicon from a TM-resistant variant. All DNA samples were digested with *Bam*HI and electrophoresed on a 0.5% agarose gel. Note: the 63-kb amplicon consists of four *Bam*HI fragments of 12, 14, 15, and 22 kb, and only the 15-kb fragment plus the 4.4-kb pBR322 (pTR15) is present in the transfectants. (B) Identical restriction patterns of pTR15 from *Leishmania* spp. and *E. coli*. Lanes 1, pTR15 plasmids from transfectants; lanes 2, pTR15 from bacteria. DNAs were digested by enzymes as indicated. pBR322 was used as the probe for Southern hybridization. ECo, *Eco*RI; Bam, *Bam*HI; Hind, *Hin*dIII. (C) Southern blot analysis of the total DNAs isolated from the transfectants electroporated with pUCP4.6. All DNAs were digested with *Bam*HI and electrophoresed in 1% agarose. Blot was probed with pUC19. Lane 1, total DNAs from parental wild-type cells; lane 2, total DNAs from transfectants electroporated with pUCP4.6; lane 3, pUCP4.6 plasmid used for transfection.

play no direct role in the expression of the NAGT gene. The functional activity of this gene was demonstrated when it was cloned into the pX vector (Fig. 7). This finding further suggests that the flanking sequences of this gene within the 4.6-kb DNA do not contribute directly to TM resistance. They probably function in regulating the expression of the NAGT gene and/or the replication or stability of the plasmids in *Leishmania* cells.

Interestingly, previous attempts to produce drug-resistant *Leishmania* spp. by transfection of wild-type cells directly with amplified DNAs from other drug-resistant variants have been unsuccessful (16, 28). Possibly, leishmanial resistance to other drugs involves multiple mechanisms, among which gene amplification is not the most favored. In contrast, DNA amplification appears to be a preferred mode of TM resistance in *Leishmania* spp. (17), which may well account for the success in our case. This preference seems to be a unique feature of *Leishmania* spp. TM resistance of CHO cells depends more often on an altered uptake of TM or NAGT gene mutation (22, 37) than on gene amplification (32).

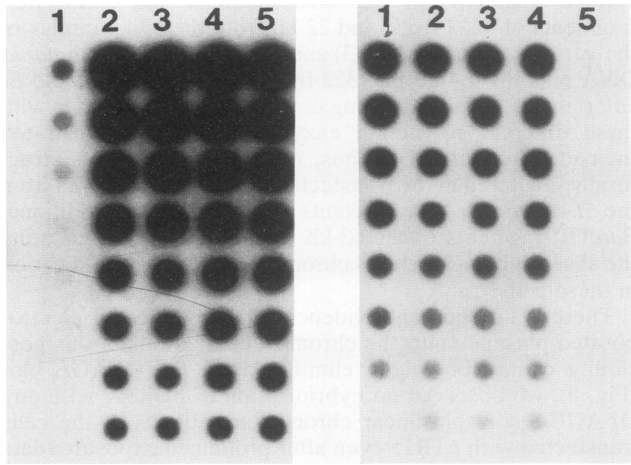


FIG. 10. Dot blot analysis of the NAGT gene amplification in TM-resistant transfectant and variant cells. Lane 1, total DNAs from the wild type; lane 2, cells transfected with pUCP4.6; lane 3, cells transfected with pTR15; lane 4, TM-resistant variants; lane 5, pTR15 plasmid DNA. Samples were each serially diluted twofold onto a nylon filter. Each series begins with 2 μg of total DNAs or 0.15 μg of pure plasmid pTR15. Left panel, hybridization with the 1.4-kb NAGT gene; right panel, hybridization with tubulin gene.

The work presented is consistent with previous findings that only a small portion of the amplified chromosomal region is responsible for drug resistance (4). In our case, the 4.6-kb DNA represents only 7 to 15% of the 30- to 70-kb amplicons found in TM-resistant variants of *Leishmania* spp. (17). Presumably, the generation of these DNA circles larger than functionally necessary is dictated by the location of the recombinogenic hot spots flanking the NAGT gene. The location of these sites in the chromosomes may vary with different *Leishmania* spp. DNA rearrangement events across these sites thus generate extrachromosomal circles of different sizes. Such hot spots have been shown to exist as inverted or direct repeats for the generation of H circles with *p*-glycoprotein genes in MTX-resistant and arsenite-resistant *Leishmania* spp. (29).

Our findings also point to the application of TM resistance as a useful genetic marker for the construction of *Leishmania* vectors. The vectors constructed so far rely on the bacterial neomycin or hygromycin resistance genes as the selective marker together with regulatory elements of other *Leishmania* genes, e.g., pR-NEO and pALT-NEO (7, 10, 11, 24, 25). The NAGT gene identified in this work provides an endogenous selective marker for transfection of *Leishmania* spp. We have indeed succeeded in expressing a *Leishmania* surface metalloproteinase gp63 by using pTR15 as the vector (27).

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